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# Crystallization and preliminary X-ray analysis of a bacteriophage T4 primase fragment

**Ingo P. Korndörfer, Jeffrey Salerno, Debra Jing and Brian W. Matthews***

Institute of Molecular Biology, Howard Hughes Medical Institute and Department of Physics, 1229 University of Oregon, Eugene, Oregon 97403-1229, USA

Correspondence e-mail: brian@uoxray.uoregon.edu

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The primase from bacteriophage T4 is a single-stranded DNA-dependent RNA polymerase that is one of the seven proteins that constitute the DNA-replication machinery of bacteriophage T4. In an attempt to crystallize the protein, a number of variants were generated. One such construct, which includes the C-terminal region (residues 196–340), gave four different crystal forms which diffract in the 3.5–6.0 Å resolution range.

## 1. Introduction

The bacteriophage T4 DNA-replication machinery consists of seven phage-encoded proteins which form the minimum functional complex to meet the requirements for performing coordinated leading- and lagging-strand DNA synthesis. Two of the seven components, the products of genes 41 and 61 (gp41 and gp61), form the primosome complex. The coupling of gp61, a primase, and gp41, a helicase, within the primosome is essential to permit effective Okazaki fragment synthesis. Gp61 is a protein of 343 amino acids with a molecular weight of 38 kDa. At very high concentrations, gp61 alone catalyzes predominantly the synthesis of template-dependent dimer sequences pppApC and pppGpC. Addition of the gp41 helicase to gp61 leads to formation of the primosome, greatly increasing the rate of primer synthesis and changing the product distribution to predominantly pentamers, which are biologically active. Gp41, which functions as a ring-shaped hexamer, has a single-stranded DNA-stimulated GTPase/ATPase activity and a 5′ to 3′ DNA unwinding activity which are stimulated by interactions with gp61 (Liu & Alberts, 1981; Venkatesan *et al.*, 1982; Hinton & Nossal, 1985; Cha & Alberts, 1986; Dong *et al.*, 1995).

## 2. Subcloning, overexpression and purification

### 2.1. Subcloning

In an attempt to obtain crystals of the gp61 primase from bacteriophage T4, an array of different expression constructs (Table 1) was created by PCR methods (Mullis *et al.*, 1986). The starting point was the vector pDH911 (Hinton & Nossal, 1985). Extensive trials with the histidine-tagged protein pQE30-61 (Table 1) were carried out but did not lead to crystals. It was found that the protein undergoes proteolytic cleavage after extended storage (six weeks) at 277 K. We analyzed the resulting fragments by N-terminal peptide sequencing and created an expression construct carrying the largest resulting fragment (pIK10; Table 1). The position of the histidine tag was varied and various N- and C-terminal deletion constructs were also created (pIK11, pDJi1–4, pDJi6). Deletion of residues from the C-terminus invariably leads to formation of inclusion bodies upon overexpression in *Escherichia coli*. Addition of the histidine tag was helpful with respect to overexpression and purification, although no crystals could be obtained using any of these proteins. In the course of obtaining these expression constructs, a PCR side product of shorter length occurred. We ligated this side product into an expression vector (resulting in pDJi5) and a soluble protein of 17 kDa (SDS–PAGE) could be overexpressed in *E. coli* strain BL21(DE3). The protein was identified by DNA sequencing as a C-terminal fragment of gp61 carrying a


**Figure 1**
Peptide sequence of pDJi5 protein. Residues marked in bold (196–341) are from the native gp61 primase. Six residues at the N-terminus of pDJi5 are a cloning artefact.

**Table 1**

Expression constructs for gp61 helicase.

The first construct (pQE30-61) includes the complete 343-amino-acid protein plus a six-histidine tag at the amino terminus. The portion of the sequence included in the other constructs is shown in parentheses. All expression vectors were based on either pET15b or pET28b(+) (NEB, Beverly, Massachusetts, USA), except pQE30-61 (ampQE30, Qiagen, Valencia, California, USA). All constructs carry a mutation E211K with respect to the sequence deposited in the SWISS-PROT database (Bairoch & Apweiler, 1999; accession number P04520). Constructs which lead to crystals are marked in bold. pIK56 gave hexagonal columns and bipyramids, pIK55 gave needles and hexagonal bipyramids and columns. Crystals of pDJi5 (Fig. 2) are listed in Table 2. gp, gene product.

| Identification code | Construct |
|---|---|
| pQE30-61 | MRGSH$_6$GS–gp61 |
| pIK10 | MGH$_6$–gp61(1–223) |
| pIK11 | MGH$_6$–gp61(1–304) |
| pIK18 | MG–gp61(2–342) |
| pDJi1 | gp61(1–335)–H$_6$ |
| pDJi2 | M–gp61(21–342)–H$_6$ |
| pDJi3 | M–gp61(28–342)–H$_6$ |
| pDJi4 | gp61(1–311)–H$_6$ |
| **pDJi5** | MNEFAY–gp61(197–342)–H$_6$ |
| pDJi6 | gp61(1–334)–H$_6$ |
| **pIK55** | M–gp61(197–342)–H$_6$ |
| **pIK56** | M–gp61(191–342)–H$_6$ |

C-terminal histidine tag and six non-native residues at the N-terminus (Fig. 1).

Two further constructs (pIK55, pIK56; see Table 1) were created to investigate the influence of the non-native residues on crystallization.

In the following, we only describe the purification of pDJi5, pIK55 and pIK56, these being the proteins that gave crystals. A similar procedure was used in each case.

### 2.2. Overexpression

Bacteria were cultured in 4 l fermentors (Applikon, The Netherlands) with Luria–Bertani medium at 310 K. Protein expression was induced by addition of 1 m*M* IPTG when the cell suspension had reached an $OD_{600}$ of 0.8. Cells were harvested 3 h later by centrifugation at 9000*g* for 10 min. Cell pellets were stored at 253 K.

### 2.3. Purification

Lysis of cells was performed by resuspension in 80 ml sonication buffer (300 m*M* NaCl, 1 m*M* $\beta$-mercaptoethanol, 20 m*M* Tris–HCl pH 8.0) and sonication (Sonic Dismembrator 550, Fisher Scientific, Pittsburgh, PA; 19 mm tip, amplitude = 8, 7 min) in the presence of 1 µg l$^{-1}$ DNAase I (Boehringer–Mannheim, Germany). The lysate was cleared by centrifugation at 35 000*g* (2 × 15 min). Glycerol was added to 10%, imidazole to 5 m*M* and the lysate was applied to a Ni–NTA agarose column (Qiagen, Valencia, CA). pDJi5 protein eluted in an imidazole gradient at approximately 80 m*M* imidazole. Fractions containing pDJi5 protein were pooled, concentrated (Centriprep 10, Millipore, Bedford, MA) and applied to a Superdex-75 size-exclusion column (Amersham Pharmacia, Piscataway, NJ) using the same buffer (200 m*M* NaCl, 20 m*M* Tris–HCl pH 8.0, 10% glycerol, 1 m*M* $\beta$-mercaptoethanol). About 400 mg of pure pDJi5 protein could be obtained from a 4 l culture.

## 3. Crystallization and crystal characterization

Using sparse-matrix crystallization screens (Jancarik & Kim, 1991), several crystal forms of the pDJi5 protein could be obtained, all under similar conditions. Hanging drops contained initial protein concentrations between 10 and 20 mg ml$^{-1}$ in buffer (10% glycerol, 20 m*M* Tris–HCl, 0.3 *M* NaCl, 1 m*M* $\beta$-mercaptoethanol pH 8.0). The well solution contained 0.1 *M* Mes (pH 5.5–6.5), 25–35% PEG 200 and 2–8% PEG 3400; crystallization occurred at 277 K. Up to three different crystal forms of pDJi5 could be found in one drop. Crystal forms I, II, III and IV of pDJi5 (Table 2, Fig. 2) could be grown to a size suitable for further studies.

## 4. Data collection and analysis

Preliminary diffraction data (Table 2) were collected at room temperature on an R-AXIS IV imaging-plate area detector with Cu $K\alpha$ radiation focused through mirror optics (Molecular Structure Corporation, The Woodlands, Texas, USA). X-rays were from an RU200 rotating-anode generator (Rigaku, Tokyo, Japan). A data set was also collected from crystals of form II at the Advanced Light Source (Berkeley Laboratory, University of California at Berkeley, California, USA). Data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997; Table 3). On the basis of density calculations, we expect to find six molecules per asymmetric unit ($V_M$ = 2.5 Å$^3$ Da$^{-1}$, solvent content 50%; Matthews, 1968).

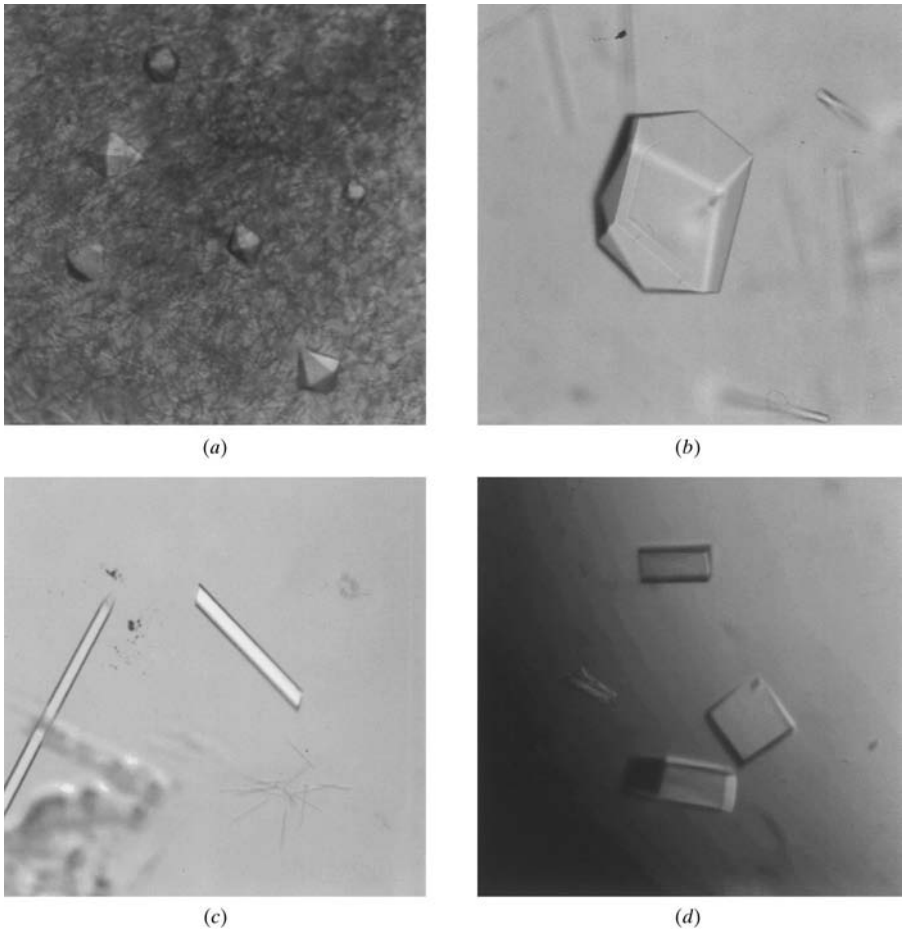


**Figure 2**
Crystal forms I (*a*), II (*b*), III (*c*) and IV (*d*) of pDJi5 protein. Crystal forms I and II usually occur together with crystal form III in the same drop. The approximate crystal sizes are listed in Table 2.

**Table 2**

Characterization of crystals from construct pDJi5.

| Crystal form | Size of crystals (mm) | Space group | Unit-cell parameters (Å) | Diffraction limit (Å) |
|---|---|---|---|---|
| I | 0.6 × 0.4 × 0.4 | $P6_{1(5)}22$ | $a = b = 161.3$, $c = 432.9$ | 4 |
| II | 0.6 × 0.5 × 0.2 | $P3_{1(2)}22$ | $a = b = 150.5$, $c = 81.9$ | 3.5 |
| III | 0.05 × 0.05 × 0.05 | $P6$ | $a = b = 147.3$, $c = 235.9$ | 5 |
| IV | 0.2 × 0.2 × 0.1 | $P222$ | $a = 118.2$, $b = 139.4$, $c = 163.4$ | 6 |

**Table 3**

Data-collection statistics of pDJi5 crystals.

| Crystal form | II |
|---|---|
| Number of measured reflections | 50205 |
| Number of independent reflections | 10228 |
| Completeness (%) | 81.9 (30.0–3.5 Å) |
| | 78.8 (3.6–3.5 Å) |
| Mosaicity (°) | 0.6 |
| Average $I/\sigma(I)$ | 3.5 |
| $R_{sym}$† | 0.12 |

† $R_{sym} = \sum |I(k) - \langle I \rangle| / \sum I(k)$, where $I(k)$ and $\langle I \rangle$ are the individual intensity measurements and the corresponding mean values, respectively. The summation is over all measurements.

The DNA primase from bacteriophage T4, like those from bacteriophage T7 and *E. coli*, contains a zinc-binding motif near the N-terminus (Mendelman *et al.*, 1991; Kusakabe & Richardson, 1996). This motif is likely to serve as a partial determinant in site-specific template recognition. Both the N- and C-termini are involved in formation of contacts with the ring-shaped gp41 T4 helicase hexamer as well as in interactions with the single-stranded DNA template (Jing, 1998). The finding that deletion of C-terminal residues renders gp61 insoluble upon overexpression in *E. coli* may indicate that the C-terminus is important for proper folding of the gp61 structure. Of the six motifs conserved throughout bacterial and bacteriophage primase domains (Ilyina *et al.*, 1987) and possibly directly involved in substrate binding, three are within the sequence encoded by pDJi5. Thus, in the absence of a three-dimensional structure for the full-length primase, a crystal structure of the pDJi5 protein would greatly contribute to our understanding of the mechanisms of molecular interaction with the other components in the primosome and catalysis of primer formation.

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